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13. ABSTRACT *(Maximum 200 Words)* The Bcl-2 protein family plays an important role in governing a cell's decision to live or die. Dysregulation of these proteins is observed in many breast cancer cases and thus it is important to understand their mechanism in order to develop new treatments. The Bcl-X_L protein structure showed a strong similarity to pore-forming bacterial toxins, suggesting that Bcl-2 protein family protein may regulate apoptosis by pore formation or membrane insertion.

Single cysteine mutants of Bcl-X_L have been generated to probe the topology of the membrane-inserted state. Preliminary results show that the BH3 domain is present on the membrane surface but it is unclear as to whether this portion of the protein is actually membrane-inserted. Labeling of these cysteine mutants with thiol-specific probes such as BODIPY also yielded results suggesting that residues in both the putative pore-forming fifth α-helix and the BH3 domain were still solvent accessible, as indicated by the ability of a fluorescence-quenching anti-BODIPY antibody to quench fluorescence in the presence and absence of lipid vesicles on which the proteins display channel-forming capability.

A new direction of work is currently being pursued which seeks to gain information regarding the relevance of Bcl-2 protein family channel formation *in vivo*. Patch-clamping of mitoplasts, which are mitochondria stripped of their outer membrane is underway and the permeability transition pore (PTP), which plays a significant role in the continuation of the apoptotic pathway has been observed in these membranes. In addition, membrane fraction of isolated mitochondria should yield more specific information regarding the localization of these proteins. Lastly, fluorescence resonance energy transfer between fluorescently tagged adenine nucleotide translocator (ANT), a component of the PTP pore, and Bcl-2 family proteins will provide in vivo observations of whether an interaction between these proteins exists *in vivo* and whether Bcl-2 family proteins can modulate ANT behavior and the PTP pore.

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FOREWORD

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INTRODUCTION

Apoptotic cell death plays an important role in tissue homeostasis by clearing aged or damaged cells thus making space for new cells. In certain situations, the Bcl-2 protein family governs a cell's decision to heed or ignore death signals and malfunctions in the Bcl-2 protein family can lead to inappropriate amounts of cell death. Bcl-2, Bcl-X_L, and Bax are each expressed in normal mammary epithelium but in a significant number of mammary carcinomas, Bax and Bcl-2 protein levels are severely depressed or absent. These depressed levels directly correlate with shorter overall survival times. These results highlight the importance of proper regulation of Bcl-2 family protein activity in the context of breast cancer.

Despite the large body of work describing the nature of protein-protein interactions that are predicted to be in important in apoptosis regulation by the Bcl-2 protein family, the biochemical function of these proteins remains unclear. The structural similarity between the Bcl-X_L structure and pore-forming bacterial toxins such as diphtheria and colicins provided a clue as to the function of the Bcl-2 protein family. This study seeks to address whether form does indeed follow function by studying if these proteins are capable of membrane insertion. The aims of this project are to determine: (i) the structural changes that occur to convert Bcl-2 family proteins from soluble to membrane-inserted proteins; (ii) which regions of these proteins participate in pore formation; (iii) identifying the residues lining the channel lumen and the relative pore size. The attainment of these goals will utilize membrane-protein biochemistry, site-directed mutagenesis, biophysical techniques-including circular dichroism and fluorescence spectroscopy- and generation of cysteine mutants for fluorescence and EPR studies.

ANNUAL SUMMARY

Sutdies on the membrane-sinserted state topology. The Bcl-X_L structure showed the protein to have two central, ~20 residue _-helices that are comprised of predominantly hydrophobic amino acids. These helices are of sufficient length to fully span a membrane bilayer whose hydrophobic cross-section is ~30 Å. Yet two helices alone should not be sufficient to create a conductive channel. This suggests that either other parts of the protein participate in channel formation by a monomer, or proteins coalesce in dimers or higher-order multimers. To address whether other parts of the Bcl-X_L protein outside of the predicted fifth and sixth _-helices, a set of six single cysteine mutants of Bcl-X_L were purified to homogeneity. Each of these cysteines provides a convenient site for attachment of thiol-specific probes, which can be used to track the environment of the cysteine residue

and gauge whether or not this region of the protein inserts into the membrane. Two experimental approaches are being followed: a) electronparamagentic resonance (EPR) studies in collaboration with Wayne Hubbell at UCLA and b) fluorometric monitoring of the thiol-reactive probe, BODIPY [*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-*s*-indacene-3-yl)methyl)iodoacetamide] and bichlorobimaine.

Preliminary EPR results using nitroxide spin-labeled wild-type Bcl-X_L and Bcl-X_L with a single cysteines at positions near the center of each helix of the Bcl-X_L structure at positions 95, 107, 124, 171, and 193 (wild-type cysteine mutated to alanine at position 151) showed that wild-type cysteine (in the center of the putative pore-forming domain helix 5) and C95, which lies at the center of the BH3 domain had significant interactions with the membrane (data not shown).

In addition, wild-type and C95 Bcl-X_L were to be labeled with BODIPY and bichlorobimaine to monitor the insertion of these proteins spectroscopically. . Bimaine is a fluorescent probe whose fluorescence is environment sensitive, displaying a blue-shifted maximum emission when the probe is in a non-polar environment, a red-shifting when the probe is exposed to the aqueous solvent and an "intermediate" shift when the probe is somewhere in between, e.g. interfacial layer at the membrane bilayer surface. Second, the Cysteine mutants were labeled with BODIPY-iodoacetamide (BODIPY=N-((4,4-difluoro-5,7-dimethyl-4-bora3a,4a-diaza-s-indacene-3-yl)methyl)iodoacetamide). The labeled proteins will be added to lipid vesicles in the presence or absence of Rabbit anti-BODIPY-FL IgG. The antibody binding quenches BODIPY fluorescence and therefore can be used as a gauge of each residue's solvent exposure, e.g. no quenching=no binding implying the probe is inaccessible to the aqueous antibody. Presumably, these assays can be applied to single-Cys mutants in other Bcl-2 family members, notably Bax and BID.

Preliminary assays with C95 and wild-type Bcl-X_L revealed that the single cysteines in this protein were labeled efficiently, even in the absence of denaturants or additional reductants. Antibodies to BODIPY were able to quench at least 50% of the fluorescence, in agreement with previously published reports (Wang et al., 1997). However, in the presence of lipids, no diminishment of quenching by the antibody could be observed (Fig. 1).

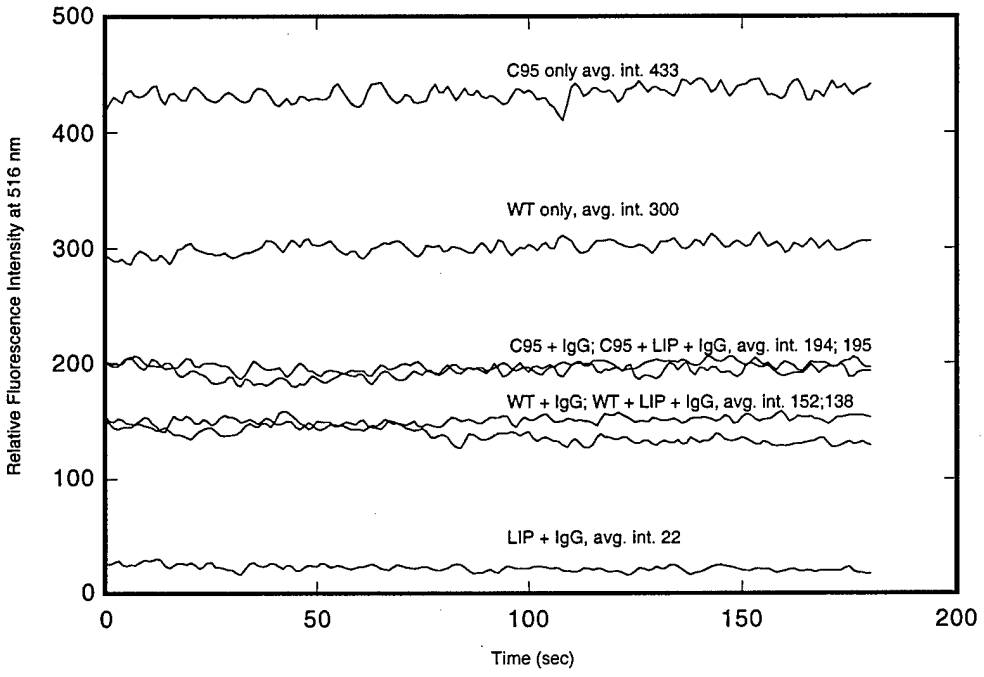

Fig. 1 Relative fluorescence intensity of BODIPY-labeled Bcl-X_L in the presence and absence of 70% DOPC/30%DOPG small unilamellar vesicles. Protein, (40 or 60 _g for C95 and wild-type, respectively) either alone or in the presence of 350_mmol lipid, and with or without 40_L _-BODIPY IgG was added to the cuvette at t=0. Measurements were made in a semi-micro cuvette (10 and 4 mm excitation and emission pathlengths, respectively). Spectra were obtained using an excitation wavelength of 488 while emission wavelength was 516 nm. Slit widths are 2.5 nm and 5 nm for excitation and emission, respectively. Background fluorescence of the buffer was negligible and not subtracted.

If the BODIPY-labeled cysteine residue had been buried within the membrane bilayer, it should be inaccessbile to solvent, and thus immune to quenching by the anti-BODIPY antibody. The result obtained is ambiguous in that it is difficult to discern whether or not the residue is completely in the solvent, or whether it is membrane-surface associated, in which case there would be membrane interaction and presumably unfolding, but not actual insertion. These experiments have been halted in favor of pursuing

collaborative work, again with Wayne Hubbell's group at UCLA and Francesa Marassi, who will conduct experiments using solid-state NMR to follow membrane insertion.

It is important to address the question of whether or not membrane insertion and channel formation is a phenomenon that occurs *in vivo*. To date, almost all information concerning the hypothesis that Bcl-2 family members are pore-forming proteins has been gathered from *in vitro* experiments. To pursue such a question will require a deviation from my plan of work, but will represent significant training opportunities.

In collaboration with Stuart Lipton's laboratory at the Burnham Institute I have begun patch clamping of mitochondrial membranes. These membranes are in the form of mitoplasts, which are mitochondria that have been stripped of their outer membrane by osmotic swelling and thus provide direct access to the inner mitochondrial membrane. Despite the small size of the mitoplasts (3_m) which makes securing membrane seals very challenging, we have already been successful in obtaining membrane seals and several seals have displayed conductances that are similar to that in previously published reports on the mitochondrial megachannel (or permeability transition pore, PTP) [Kinnally et al., 1996]. Confirmation that the observed conductances are indeed that of the mitochondrial megachannel await the addition of the PTP inibitors bongkrekic acid and atractylate, which should silence PTP activity. In addition, the patch clamping technique can be exploited for studies of channel formation by either a) addition of recombinant Bcl-2 family proteins into the pipette solution for direct application of these proteins onto mitochondrial membrane; or b) construction of plasmid vectors that contain a Bcl-2 family protein-CAAX-box hybrid protein. The CAAX box will target the Bcl-2 family protein that is encoded (Bcl-2, Bax, or Bcl-X_L) to the plasma membrane which will allow for direct patching of the plasma membrane, which is less demanding than patching of mitoplasts. Such CAAX-box transfected cells can then be compared against sham-transfected cells to look for the appearance of new channel conductances, indicative of Bcl-2 protein family membrane insertion.

Two other directions will also be pursued and though they also represent deviations from the plan of work, but have the potential to yield valuable information about the function of the Bcl-2 protein family. I plan to more carefully examine the question of localization of the Bcl-2 family proteins in the mitochondria. Using fractionation of isolated mitochondria and Western blotting using antibodies against both mitochonrial marker proteins and Bcl-2 family proteins, I hope to gain information about whether these proteins reside on the inner or outer mitochondrial membrane. To date, studies that are referenced as to localization of these proteins rely on electron micrographs to show localization.

Other plasmid constructs are currently being assembled which will tag the adenine nucleotide translocator (ANT) and several different Bcl-2 family protein members with GFP (green fluorescent protein) and RFP (red fluorescent protein). This color pair makes possible the use of fluorescence resonance energy transfer (FRET) between the two proteins which will yield information about whether the Bcl-2 family proteins serve as modulators of the mitochondrial permeability transition. This direction will allow training opportunities on a deconvolution microscope. These new directions represent exciting opportunities for both learning about Bcl-2 protein family function and expansion of my technical repertoire.

APPENDIX

Research Accomplishments:

* Fluorescent labeling of Bcl-X_L single cysteine mutants with BODIPY did not yield expected information regarding the degree of insertion of each helix of the protein.
* Development of patch clamping skills which are being used to patch clamp mitochondrial membranes which will be useful in obtaining information about Bcl-2 protein family channel formation *in vivo*
* Development of microscopy skills on deconvolution fluorescent microscope which can be used to gather information regarding interactions between Bcl-2 family proteins and mitochondrial membrane proteins *in vivo.*

Reportable outcomes:

Schendel SL, and Reed JC. Methods for assay of Bcl-2 family protein ion channel activity. *Meth. Enzymol. (in press).*

Deveraux QL, **Schendel SL**, and Reed JC. Anti-apoptotic proteins: The BCL-2 and IAP protein Families. *Cardiac Clinics (in press)*